STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

A. Purpose:

This is an optional EPA-approved procedure for evaluating tuberculocidal activity based upon the results of studies reported by Ascenzi, J.M. et al., Appl. Environ. Microbial. 93:2189-2192, 1987. The procedure may be used to establish the effectiveness of a product claiming tuberculocidal efficacy.

B. Summary of Method:

A dilution of a culture of *Mycobacterium bovis* BCG is made into the test product at the desired temperature; this is followed by periodic sampling, neutralization of the disinfectant, and filtration. The filter containing the surviving bacterial cells is then placed on the surface of recovery medium and incubated for 15-20 days. Colonies appearing on the filter/culture medium are counted and survival curves are constructed to determine the tuberculocidal activity of the disinfectant. The minimum exposure time that can be claimed for efficacy is reported as the time required to kill all but one bacterial cell or less.

C. Media and Reagents:

1. Modified Proskauer-Beck medium - Dissolve each of the following in the following order in 900 ml of Milli-Q H_2O: 2.5 g KH_2PO_4, 5.0 g asparagine, 2.5 g Mg citrate and 20 ml glycerol. Adjust to pH 7.2-7.4 with 1 N NaOH. Autoclave at 121°C for 15 min. Allow to cool and aseptically add 100 ml of sterile 0.6% $MgSO_4.7H_2O$ in Milli-Q H_2O.

2. Modified Proskauer-Beck medium with Tween 80 - Mix 1 ml of Tween 80 into 1 L of medium (C.1). Autoclave as described above.

3. Mycobacteria 7H11 agar - Dissolve 21 g Bacto-Mycobacteria 7H11 agar (Difco) in 900 ml of Milli-Q H_2O containing 5.0 ml of glycerol. Heat to boiling to dissolve the medium completely. Autoclave at 121°C for 15 min. To each 450 ml of sterile medium cooled to about 45-55°C, add

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

50 ml of Bacto-Middlebrook OADC enrichment under aseptic conditions. Dispense 10-15 ml each into individual 60 x 20 mm Petri plates.

4. Buffered Gelatin - Dissolve 2.8 g $NaH_2PO_4 \cdot H_2O$ in a final volume of 100 ml Milli-Q H_2O. Dissolve 5.4 g $Na_2HPO_4 \cdot 7H_2O$ (or 2.8 g Na_2HPO_4) in a final volume of 100 ml Milli-Q H_2O. Mix 33 ml of the NaH_2PO_4 solution with 67 ml of the Na_2HPO_4 solution, adjust to pH 7.1 and dilute to 200 ml with Milli-Q H_2O. Dissolve 2.0 g of Bacto-gelatin (Difco) in the phosphate buffer. Autoclave at 121°C for 15 min.

5. Saline solution - Dissolve 8.5 g of NaCl in Milli-Q water and adjust the volume to 1 liter. Autoclave at 121°C for 15 min.

6. Saline - Tween 80 solution - Dissolve 1 ml Tween 80 in 1 L saline solution (C.5). Autoclave at 121°C for 15 min.

7. Phenol stock solution 4% - Dissolve 4 g phenol crystals (USP) in Milli-Q H_2O; then adjust to a final volume of 100 ml.

8. Phenol test solution - Make a 5-fold dilution of the phenol stock solution (C.7). This will result in a final 0.8% phenol solution.

9. Sterile normal horse serum without preservatives, if necessary.

10. Neutralizer - A neutralizer appropriate for the active ingredient should be used. Static activity controls should be done in order to verify the neutralizer capability. Also, it may be required to demonstrate that the neutralizer is not active against the test organism at concentrations employed in this method.

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

a. Lecithin - Tween 80 neutralizer. For use with <200 ppm quaternary ammonium compounds or phenolics.

1. Phosphate buffer stock solution 0.25 M. Dissolve 34.0 g of KH_2PO_4 in 500 ml Milli-Q H_2O, adjust to pH 7.2 with 1N NaOH and dilute to 1L.

2. Neutralizer stock solution. Mix 40 g Asolectin, 280 ml Tween 80, and 1.25 ml phosphate buffer (A.10.a.1); dilute with Milli-Q H_2O to 1L and adjust to pH 7.2. Dispense in 100 ml portions and autoclave for 20 min at 121 °C.

3. Neutralizer blanks. Mix 100 ml neutralizer stock (A.10.a.2), 25 ml phosphate buffer (A.10.a.1) and 1675 ml of Milli-Q H_2O. Dispense 2 ml into tubes and autoclave at 121 °C for 20 min.

b. Sodium metabisulfite. Neutralizer for use with glutaraldehyde. On the day of assay, prepare a solution of $Na_2S_2O_5$ in Milli-Q water in a volumetric flask. The actual concentration of $Na_2S_2O_5$ will depend upon the concentration of glutaraldehyde to be neutralized. Sterilize the solution by filtration and dispense 2 ml volumes into sterile tubes.

11. Synthetic hard water, if necessary. Prepared according to QA/SOP No.: MV-54.

D. Materials:

1. Incubator, nonhumidified.
2. Incubator, humidified
3. Tissue homogenizer. - glass with Teflon pestle.
4. Bacteriological filters and filter holders. - 47 mm diameter filter with 0.45 μm average pore size.

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

5. Roller culture apparatus with 2 L cell culture bottle.
6. Water bath.
7. Disposable petri plates - 60 x 20 mm.
8. Plastic bags.

E. Culture Preparation:

1. Stock culture - Use *Mycobacterium bovis* BCG strain TMC 1028. This strain was obtained from Dr. Joe Ascenzi, Surgikos, Arlington, TX (Surgikos strain No. 899-60). The SRI strain No. is 1202; the ATCC designation for this strain is 35743.

A lyophilized culture is inoculated into 10 ml of culture medium (Modified Proskauer-Beck) and incubated at 37°C until a pellicle forms. A loopful of the pellicle is then transferred onto the surface of 10 ml of culture media (C.2) (Modified Proskauer-Beck with Tween 80). Incubate at 37°C until the culture is turbid. Transfer the 10 ml of culture to 100 ml of medium (C.2) in a 250 ml flask. Incubate for 5-7 days. Shake the flask daily to aerate. Add 40 ml of the subculture to 400 ml of culture medium (C.2) in a 2 L roller bottle and incubate for 15-20 days at 37°C, rolling slowly. Harvest the cells when the culture reaches 1-5 x 10^8 CFU/ml; an absorbance at 500 nm of 0.7-0.8 (13 mm light path) with a Bausch & Lomb Spectronic 20 spectrophotometer approximates the required cell density. Add additional sterile Tween 80 (1 ml/L) to the culture one day prior to harvest. Dispense in cryovials (1-2 ml/vial) and freeze at -70°C. Thaw a vial of frozen stock culture in cold tap water, dilute and plate on medium (C.3) to determine the number of viable CFU/ml after freezing.

2. Test Culture - Remove and thaw a vial of stock culture in cold tap water at room temperature. Add an equal vol of buffered gelatin to the cell suspension and homogenize in ice with a Potter-Elvehjem apparatus with a Teflon pestle for 1 min. Allow the homogenate to settle for 15 min, then adjust the homogenized cell suspension with saline/Tween 80 solution to approximately 10^7 CFU/ml using the plate count

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

data obtained previously (E.1). Prepare duplicate 10-fold serial dilutions of the test culture in saline; filter and plate, also in duplicate, one ml of the 10^{-5} and 10^{-6} dilutions as described in section F. Use these data to calculate S_o as described on the attached Calculation of S_o Data Sheet.

F. Test Method - Disinfectant:

Dilute the test disinfectant in Milli-Q H_2O according to label instructions. If label claims are to include organic soil tolerance and/or hard water tolerance, the dilution must be prepared in water containing 5% normal horse serum and/or water of appropriate hardness.

Let 4 tubes (25 x 150 mm) containing 9 ml of the use-dilution germicide sample to be tested come to the specified exposure temperature in a water bath, then add 1 ml of the test culture (E.2) to each tube. If more than 4 time points are to be sampled, prepare a larger volume of test sample using the proper ratio of germicide to cells. Mix by swirling and at appropriate time intervals, remove 2 ml amounts of the germicide-cell suspension and add the suspension directly to 2 ml of appropriate neutralizer (C.10). Mix thoroughly and, within 5 minutes, make ten-fold dilutions of neutralized sample in saline (C.5) dilution blanks. Begin filtering appropriate dilutions starting with the highest dilution. First add 10-20 ml of sterile saline onto the membrane in the filter holder. Then pipet 1 ml of a sample dilution into the saline on the surface of the filter. Turn on the vacuum and wash the filter and holder with at least 50 ml of saline. Aseptically remove the filter from the filter holder and place it on the surface of the recovery medium (C.3). Incubate the plates in plastic bags for 15-20 days at 37°C in a humidified incubator. Colonies should be counted using a dissecting microscope and lateral lighting. Record results on the appropriate data sheet.

Survival curves should be constructed to determine the tuberculocidal activity of the test sample. Data should be plotted as $\log_{10} S/S_o$ vs. time. S_o is calculated by determining the colony forming units (CFU)/ml of the test organism in the germicide sample at Time 0; S is the viable count at each time point for each replication. If replicates are assayed on different days, S_o must be determined for each day's test culture.

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

Survival curves should be derived from the average of at least four replicates from each time point so that upper 95% confidence limits can be calculated. The value for each upper 95% confidence limit is calculated by multiplying the standard deviation of the mean of S/S_o for each time point by 1.96 and adding that value to the mean. The minimum time that can be claimed for efficacy is determined by finding the point at which the upper 95% confidence limit curve intersects the X-axis at a time point representing the probability of one survivor (i.e. 1 divided by S_o). If the data show at least a four $\log_{10}$ kill of the initial cell population but the survival curve does not intersect the one survivor line on the graph, the minimum time that can be claimed is determined by extrapolating the upper 95% confidence limit curve to the one survivor line using the last two points on the 95% confidence limit curve as a basis for the extrapolation.

The time established for tuberculocidal efficacy claims must be in five minute increments. If the one survivor time point is a time other than that falling on a five minute increment, the claim will be established by rounding up to the next five minute increment.

G. Test Method - Phenol Control:

Let 1 tube containing 9 ml of 0.8% phenol (C.8) come to temperature in a 25°C H_2O bath and add 1 ml of the test culture (E.2). Mix by swirling and at 20 min, remove a 1 ml sample and add directly to an equal volume of lecithin-Tween 80 neutralizer (C.10.a). Mix thoroughly and, within 5 minutes, make ten-fold serial dilutions of the neutralized sample in saline. Filter and plate 1 ml of the 10^{-3} and 10^{-4} dilutions. Prepare four replicates of this control and average the plate counts of the replicate exposures. The test culture should show no less than a 0.5 $\log_{10}$ kill and no more than a 1.0 $\log_{10}$ kill after a 20 min exposure at 25°C. Record results on the appropriate data sheet. The CFU/ml at the "zero" exposure time is estimated to be the CFU/ml of the test culture divided by 10.

H. Test Method - Static Control:

Let duplicate tubes containing 4.5 ml of the use-dilution germicide to be tested come to the specified exposure temperature in a H_2O bath and then add 5.0 ml of neutralizer to each tube. Mix by swirling, then

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

STANDARD OPERATING PROCEDURE

QA/SOP No.: MV-59.2

Procedure Title: Quantitative Suspension Method for Determining Tuberculocidal Activity

add 0.5 ml of the test culture, mix again and let stand at room temperature. Remove 1 ml after a 5 min exposure time and prepare appropriate ten-fold dilutions in saline. Filter and plate 1 ml of the 10^{-3} and 10^{-4} dilutions in duplicate as described above. Record the results on the appropriate data sheet. The CFU/ml at the "zero" exposure time is estimated to be the CFU/ml of the test culture divided by 20.

I. Test Method - Neutralizer Toxicity Control:

Add 0.5 ml of the test culture (E.2) to each of duplicate tubes containing 9.5 ml of neutralizer at room temperature. Mix and hold at room temperature for 5 minutes and remove 1.0 ml and make ten-fold dilutions into saline. Filter and plate 1.0 ml of the 10^{-3} and 10^{-4} dilutions in duplicate as stated in Section F above. Record the results on the appropriate data sheet. The CFU/ml at the "zero" exposure time is estimated to be the CFU/ml of the test culture divided by 20.

J. Attachments

Media and Reagent Preparation	(1 page)
Quantitative Tuberculocidal Activity Data Sheet	(1 page)
Calculation of S_o Data Sheet	(1 page)
Calculations for Preparing Survival Curves	(1 page)
Phenol Resistance Data Sheet	(1 page)
Static Control Data Sheet	(1 page)
Neutralizer Toxicity Control Data Sheet	(1 page)

Approved by:

William M. Shannon

Director, Microbiology Research Department and
Head, Virology and Biotechnology Divisions

December 18th, 1992

Effective Date

Attachment to QA/SOP No.: MV-59.2
Media and Reagent Preparation

Description _____ Date _____

Assigned Lot No. _____ Tech _____

Expiration Date _____

Store at _____

Balance # _____

Component	Tare	Amount	Supplier	Lot No.	Exp. Date

Working Notes:

Attachment to QA/SOP No.: MV-59.2
Quantitative Tuberculocidal Activity Data Sheet

Date: ____________________
Technician: ____________________
Internal Study I.D. No.: ____________
Test Product/Lot No.: ____________________

Project No.: ____________
Dates of Incubation: ________ to ______
Notebook No.: __________

Assay Code	Time of Exposure	CFU at a dilution of 10^{-1}				CFU at a dilution of 10^{-3}				CFU at a dilution of 10^{-5}				CFU/ml*			
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D

Working Notes:

Attachment to QA/SOP No.: MV-59.2
Calculation of S_o Data Sheet

Date: _____

Technician: _____

Internal Study I.D. No.: _____

Project No.: _____

Dates of Incubation: _____ to _____

Microorganism: *M. bovis* BCG SRI 1202

Plate Count of Test Culture:

Assay Code	CFU/plate at a dilution of 10^{-5}	CFU/plate at a dilution of 10^{-6}	Avg. CFU/ml/ replicate[1]	Avg. CFU/ml[2]
_____	_____	_____	_____	_____
_____	_____	_____	_____	

Calculation of S_o:

S_o = Avg. CFU/ml of Plate Count ÷ 10. A 10-fold dilution of test culture in the test article is assumed.

1. The numbers circled were used to determine CFU/ml by the following formula: average CFU/plate X dilution factor of dilution plated = CFU/ml.

2. Average of replicate CFU/ml.

Attachment to QA/SOP No.: MV-59.2
Calculations for Preparing Survival Curves (page 2)

Date: _____
Technician: _____
Internal Study I.D. No.: _____
Test Product/Lot No.: _____

Time of Exposure (min)	S/S_o 1	2	3	4	Mean $S/S_o \pm$ S.D.	Upper 95% CL

Working Notes:

Attachment to QA/SOP No.: MV-59.2
Phenol Resistance Data Sheet

Date: _____

Technician: _____

Internal Study I.D. No.: _____

Project No.: _____

Dates of Incubation: _____ to _____

Microorganism: _____

Assay Code	Time of Exposure (min)	CFU at a dilution of 10^{-3}	CFU at a dilution of 10^{-4}	CFU/ml[1]	Avg. CFU/ml	Log_{10} reduction in population[2]
____	20	____	____	_____	_____	_____
____	20	____	____	_____		
____	20	____	____	_____		
____	20	____	____	_____		

Comments:

1. The numbers circled were used to determine CFU/ml by the following formula: CFU/plate X dilution factor of dilution plated X 2, to account for the initial two-fold dilution with neutralizer.

2. Calculate log_{10} reduction as follows: log_{10} CFU/ml (S_o) - log_{10} CFU/ml (phenol control) = log_{10} reduction in CFU.

Attachment to QA/SOP No.: MV-59.2
Static Control Data Sheet

Date: _____

Technician: _____

Internal Study I.D. No.: _____

Project No.: _____

Dates of Incubation: _____ to _____

Neutralizer/Lot No.: _____
Test Product/Lot No.: _____

Assay Code	Time of Exposure (min)	CFU at a dilution of 10^{-3}	CFU at a dilution of 10^{-4}	Average CFU/ml/replicate[1]	Average CFU/ml[2]
____	5	_____	_____	_____	_____
____	5	_____	_____	_____	

Neutralizer/Lot No.: _____
Test Product/Lot No.: _____

Assay Code	Time of Exposure (min)	CFU at a dilution of 10^{-3}	CFU at a dilution of 10^{-4}	Average CFU/ml/replicate[1]	Average CFU/ml[2]
____	5	_____	_____	_____	_____
____	5	_____	_____	_____	

Working Notes:

1. The numbers circled were used to determine CFU/ml by the following formula: average CFU/plate X the dilution factor of the dilution plated.

2. Average of replicate CFU/ml.

Attachment to QA/SOP No.: MV-59.2
Neutralizer Toxicity Control Data Sheet

Date: _____

Technician: _____

Internal Study I.D. No.: _____

Project No.: _____

Dates of Incubation: _____ to _____

Neutralizer/Lot No.: _____

Assay Code CFU/ml[2]	Time of Exposure (min)	CFU at a dilution of 10^{-3}	CFU at a dilution of 10^{-4}	Avg. CFU/ml/ replicate[1]	Avg.
_____	5	_____	_____	_____	_____
_____	5	_____	_____	_____	

Comments:

1. The numbers circled were used to determine CFU/ml by the following formula: average CFU/plate X dilution factor of dilution plated.

2. Average of replicate CFU/mL